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Cloning, Expression, and Immunogenicity of the Assembly Protein of Varicella-Zoster Virus and Detection of the Products of Open Reading Frame 33

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Herpesviruses produce assembly proteins (AP) that act as scaffolding proteins for the assembly of the viral capsids. The products of the *assemblin* gene, which encodes both maturational protease and AP, have been established for herpes simplex virus type 1 (HSV-1) and human cytomegalovirus (CMV). We cloned an inframe ORF (encoding amino acids 304–605), found within the ORF 33 assemblin gene of VZV, into a yeast expression vector. The 34-kDa AP was expressed as a fusion protein with the particle-forming Ty p1 protein, resulting in high-level production of hybrid AP-virus-like particles (AP-VLPs). When AP-VLPs were injected into mice and rabbits, antibodies were produced that reacted with, but that did not neutralise, native VZV. Three of four inbred strains of mice immunised with AP-VLPs produced a VZV-specific T-cell response. The mouse and rabbit sera reacted with six bands on native VZV by Western blot analysis. The dominant bands were found at 34 and 38 kDa. Bands were also seen at 66, 63, 41, and 31 kDa. The 38-kDa protein may represent the mature AP derived from the 41-kDa precursor AP, itself the release product from the full-length 66-kDa assemblin. The 34-kDa protein probably represents the product of the inframe co-translational gene within ORF 33 encoding amino acids 304–605. The genetic organisation and proteolytic maturation of VZV assemblin are, therefore, analogous to those of other herpesviruses.
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KEY WORDS: HSV; assembly proteins; CMV

INTRODUCTION

Varicella-zoster virus (VZV) is the causative agent of chickenpox and shingles. It is a member of the Alphaherpesviridae family, and many proteins of VZV have homologues in herpes simplex virus (HSV) and other herpesviruses [Davison, 1991]. The assembly proteins (APs) of herpesviruses are known to play an important role in assembly of viral capsids [Rixon, 1993; Harper, 1994]. APs are produced from open reading frames (ORFs) which contain one or more smaller inframe 3′-cotranslational ORFs encoding overlapping proteins with identical C-terminii. The full-length protein or assemblin contains both the assemblin protease and its substrate, AP. In the case of HSV-1, two proteins are produced from within the U_L26 gene, a full-length product (66-kDa assemblin containing the protease) and a C-terminal precursor AP. A similar pattern is found with EBV and in the case of HCMV, four proteins may be produced [Welch et al., 1991]. The maturational protease releases itself from the full-length assemblin protein and converts the released precursor AP to mature AP by cleavage of a short fragment from the C-terminus at a highly conserved site. This fragment is also removed from the shorter assemblin gene products. This gene transcription pattern and subsequent maturational proteolysis events leads to multiple protein species, some of which may be phosphorylated [Harper, 1994].

VZV also produces APs, which are associated with the viral nucleocapsid and with the nucleus of infected cells. The VZV assembly proteins resolve as two major bands on sodium dodecyl sulfate (SDS)-polyacrylamide gels, the p32/p36 complex [Friedrichs and Grose, 1986]. Two minor proteins at 34 and 38 kDa are also detected. The p36 and p38 proteins are phosphorylated [Harper et al., 1995) and all forms resolve as multiple spots by

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two-dimensional electrophoresis [Ashcroft et al., 1993]. VZV gene 33 has been identified as the homologue of the HSV assemblin gene (U_L26) [Davison and Scott, 1986], and the full-length protein (605 amino acids) contains the protease release site at residue 236 (VYLQA-STGYG), a protease cleavage site (NAVEA-SSKAP) 27 amino acids from the C-terminus and conserved regions in the N-terminal half consistant with protease activity. There are multiple possible initiation sites within the ORF 33, including an internal methionine at codon 305.

As with other herpesvirus assembly proteins, the p32/p36 proteins are immunogenic, particularly for IgM antibodies [Harper and Grose, 1989]. Cell-mediated immune responses are vital for protection against varicella and zoster infections [Arvin, 1992], and T-lymphocyte responses have been detected against the immediate early protein, IE62, in infected and vaccinated individuals [Giller et al., 1989; Arvin, 1994] and immunisation with IE62 protects guinea pigs against challenge [Sabella et al., 1993]. By analogy with many other viruses, T-lymphocyte responses to nucleoproteins such as AP may be important for the control of infection, particularly CD8+ cytotoxic T cells. T-cell responses of AP of VZV have not been studied to date.

In order to investigate the immunogenicity of AP and to define the transcription products and proteolytic products of the assemblin protein of VZV, we have cloned and expressed the putative AP within ORF 33 and used this to generate antisera to identify the products of ORF 33 in VZV-infected cells. A recombinant particulate antigen presentation system was used based on the self-assembling properties of the carrier protein p1, encoded by the *TYA* gene of the yeast retrotransposon, Ty [Adams et al., 1987a,b]. Overexpression of hybrid *TYA:*antigen genes in yeast results in the production of p1 fusion protein, which assembles into virus-like particles (VLPs). VLPs carrying viral proteins are potent inducers of humoral and cellular antiviral responses [Griffiths et al., 1991; Harris et al., 1992; Martin et al., 1993].

MATERIALS AND METHODS

Viral Antigen

VZV antigen was prepared from a clinical isolate (H-551). The virus was propagated in MRC-5 human fibroblast cells as described previously [Harper et al., 1988]. Cells were inoculated at a ratio of 1 infected cell to 10 uninfected cells and incubated for 3–4 days. The confluent cell monolayer was then rinsed with phosphate-buffered saline (PBS) and scraped into PBS. The cells were pelleted by centrifugation (800*g* for 15 min at 4°C) and the virus extracted by resuspending the cell pellet in 0.1 M glycine/0.1 M NaCl buffer (pH 9.6) and incubating for 1 hr at room temperature, followed by ultrasonication. The cell lysate was clarified by centrifugation (300*g*, 10 min) and the supernatants stored at −70°C. A negative control cell lysate antigen was prepared in the same manner using uninfected cells.

Amplification of VZV Assembly Protein Gene by PCR

A gene fragment encoding the C-terminal half of ORF 33 (codons 304–605) was amplified from genomic DNA derived from VZV isolate H-551 by PCR. Codon 304 is an internal methionine. The reactions were carried out in a total volume of 50 μl, using 1 μg of each primer, 1 mM each dNTP (Pharmacia, St. Albans, Herts, UK) and 2 units of $Vent_R$DNA polymerase (New England Biolabs, Hinchin, Herts, UK) in a buffer containing 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris–HCl (pH 8.8), 2 mM $MgSO_4$. The PCR oligonucleotide primers were designed to incorporate an 8-base pair (bp) "stuffer fragment" and a *Bgl*II restriction endonuclease site. Amplification was carried out with an initial round of denaturation at 95°C (5 min) addition of $Vent_R$DNA polymerase, 60°C annealing (1 min) and 72°C extension (3 min), followed by 30 repeated cycles of 95°C (1 min), 60°C (1 min) and 72°C (3 min) with a final 35-min step at 72°C. The PCR product was detected by agarose gel electrophoresis and ethidium bromide staining.

Construction of Plasmids

The amplified DNA was digested with *Bgl*II and purified from agarose using GENECLEAN II (BIO101). The vector pOGS1215 was generated by inserting the purified DNA into the *Bgl*II site of the modified transfer vector pSP64 (Promega, Southhampton, Hants, UK). Recombinant plasmids were identified by restriction analysis and purified using QIAGEN columns (Hybaid). pOGS1215 was digested with *Bgl*II and the fragments separated by gel electrophoresis. The DNA fragment encoding AP was transferred by electroelution onto a DEAE membrane (Schleicher and Schuell Ltd; Kingston, Surrey, UK 0.45 μm) and recovered by elution into high salt buffer. The purified fragment was then ligated to the truncated *TYA* gene of the yeast/*Escherichia coli* shuttle vector pOGS40 resulting in the vector pOGS1217. The expression of this vector in yeast is under the control of a PGK-GAL hybrid-inducible promoter [Adams et al., 1991]. The nucleotide sequence of the constructions were confirmed using Sequenase (United States Biochemical, Amersham, Bucks, UK).

Production of Hybrid AP-VLPs in Yeast

Saccharomyces cerevisiae strain MC-2 was transformed with pOGS1217, as previously described [Burns et al., 1991]. Single colonies were purified by streaking onto fresh plates. The transformed cells were grown selectively in 50 ml SC-glc medium at 30°C for 24 hr with agitation. This was used to innoculate 500ml SC-glc medium supplemented with 10 g/L galactose until a cell density of 4–6 × 10^7/ml was reached (~18 hr). These cells were then used to innoculate a 12 L fermentation vessel. The cell harvest was homogenised and the VLPs purified from the clarified homogenate by fractionation on a 35–60% sucrose gradient, followed by size-exclusion chromatography as

previously descried [Burns et al., 1991]. This material is generally between 80–95% pure.

SDS–PAGE and Western Blot Analysis

Denaturing SDS–PAGE was carried out according to Laemmli [1970], using the Min-protean II (BioRad, Hemel Hempstead, Herts, UK) apparatus. Gels contained 12.5% acrylamide/bisacrylamide, ratio 37:1 (Sigma, Poole, Dorset, UK; A-6050). The samples were diluted (1:1) in loading buffer and boiled for 3–5 min. The denatured samples were applied on to the gel at concentrations of 0.5–5 μg/lane. Electrophoresis was carried out at 200 V. The gel was stained with Coomassie blue and then de-stained in methanol (20% v/v) and glacial acetic acid (10% v/v). Molecular-weight markers were used to facilitate the calculation of the molecular weights for the unknown proteins.

Proteins separated by SDS–PAGE were transferred on to a nitrocellulose membrane (Schleicher and Schuell; 0.45 m; BA85, Kingston, Surrey, UK) using a wet Western blotter (Mini-protein II, BioRad). A constant current of 100 V for 1 hr was applied to transfer the proteins. The membranes were then thoroughly washed with distilled water and incubated with blocking buffer [PBS containing 5% (w/v) skimmed milk (Marvel)] for 1 hr at room temperature (RT). The membranes were washed three times (5 min each wash) with PBS/Tween 20 (0.05% v/v) and incubated for 1 hr at RT with test sera or monoclonal antibody (mAb) diluted in blocking buffer. The membranes were washed, as before, and incubated with peroxidase-labeled antihuman, antimouse, or antirabbit IgG conjugate (Sigma) diluted 1:500 in blocking buffer. The membranes were washed and developed with a solution containing nickel chloride (2% w/v, 0.3 ml), 3,3′-diaminobenzidine (1 mg/ml, 1.5 ml), hydrogen peroxide (0.002% v/v, 30 μl) in PBS (13.2 ml). The reaction was stopped with distilled water and the membranes were dried in the dark.

Immunisation Protocols

A group of 11 Swiss white mice (female, 5–8 weeks old) were immunised intramuscularly (i.m.) with a dose of 100 μg/mouse of AP-VLPs adjuvanted with alum (aluminum hydroxide; protein-to-aluminum ratio 1:5; Alhydrogel 85, Superfos Ltd., Vedbaer, Denmark). Mice were boosted on week 4 and bled on week 6. A Dutch dwarf rabbit (male, 6–8 weeks old) was immunised with a dose of 400 μg (i.m.) of AP-VLPs adjuvanted with alum (protein-to-A1 ratio: 1:5). The rabbit was boosted on weeks 3 and 8 and was bled at week 10.

Enzyme-linked Immunosorbent Assay

Ninety-six well plates (Nunc Immunoplate II, GibcoBRL, Paisley, Scotland, UK) were coated with VZV antigen (5 μg/ml) diluted in coating buffer (carbonate/bicarbonate buffer, 0.05 M, pH 9.6) and incubated overnight at 4°C. The plates were washed 3× with PBS/Tween 20 (0.1%) and then incubated with twofold dilutions of sera (1:100 starting dilution) in duplicate for 2 hr at RT. The plates were washed as before. The bound antibodies were detected with either horseradish peroxidase (HRP)-labeled antimouse (Sigma A9044) or antirabbit (Sigma A0545) IgG conjugates (2 hr at RT). Serum samples and conjugate solutions were prepared in blocking buffer [PBS/Tween 20 (0.05% v/v)/casein (2% w/v)]. The plates were washed as above, and a fresh substrate solution containing 2,2′azino-bis (3 ethylbenz-thiazoline-6-sulfonic acid) (ABTS) and H_2O_2 in citrate/phosphate buffer (0.05 M, pH 4.2) was prepared and added immediately to the plates. After 15 min, the reaction was stopped by addition of NaF (2.5% w/v). The optical density (O.D.) values were measured at 405 nm. The titres of the sera were arbitrarily determined as the reciprocal of the dilution with an O.D. value of 0.2 (end-points) after subtracting the nonspecific reactivity with controls.

Neutralisation Assays

Cell-free VZV was obtained from VZV-infected MRC-5 fibroblast cells after 2–3 days infection, when the cytopathic effect was 10–30%. This was then sonicated 3 × 2 min on ice and clarified at 1500 g for 15 in. The cell-free virus prepared contained 200–800 PFU/ml. Pooled sera were heat inactivated at 56°C for 30 min. Virus (100 μl) was incubated with serial dilutions of sera (40 μl) at 37°C for 30 min. Where complement-dependent neutralisation was assayed, six haemolytic units of rabbit-derived complement serum were added (20 μl). Confluent monolayers of a human melanoma cell line (Mewo) were infected with the serum plus virus mixture in 24-well plates for 1 hr. After adsorption, the cells were washed with PBS and overlaid with maintenance medium solidified with agarose (0.15% w/v). Cultures were incubated at 32°C for 8–9 days, then fixed with formal saline [formaldehyde (10% v/v) in PBS]. The number of plaques was counted after staining with crystal violet. The antibody neutralising titre was defined as the dilution in which the plaque number was reduced to 50% of the mean value of the control (without serum).

Lymphocyte Proliferation Assays

Four different strains of inbred mice (BALB/c, C57BL/6, CBA, and B10.G), with H-2 haplotypes d, b, k, and q, respectively, were immunised with AP-VLPs (100 μg/mouse in alum, subcutaneously). At 7–14 days later, draining lymph nodes were removed and a single cell suspension was prepared in RPMI 1640 medium (GibcoBRL Ltd., Paisley, Scotland, UK) containing fetal calf serum (Sigma; 10% v/v) and L-glutamine/penicillin/streptomycin solution (Sigma; 1% v/v). The antigens (VZV lysate, mock lysate, control-VLPs or AP-VLPs) were tested at the optimal concentration (10 μg/ml) in 96-well round-bottom microtitre plates (Costar 3799). The mitogen, Con A (Sigma) at 5 μg/ml was used as a positive control. The cells in culture medium alone were negative controls (background). Lymph node cells (5×10^5/well; 100 μl/well) were cultured with each antigen or mitogen (100 μl/well) in triplicate for 6 days at 37°C (5% (v/v) CO_2; 95% (v/v) humidified incubator).

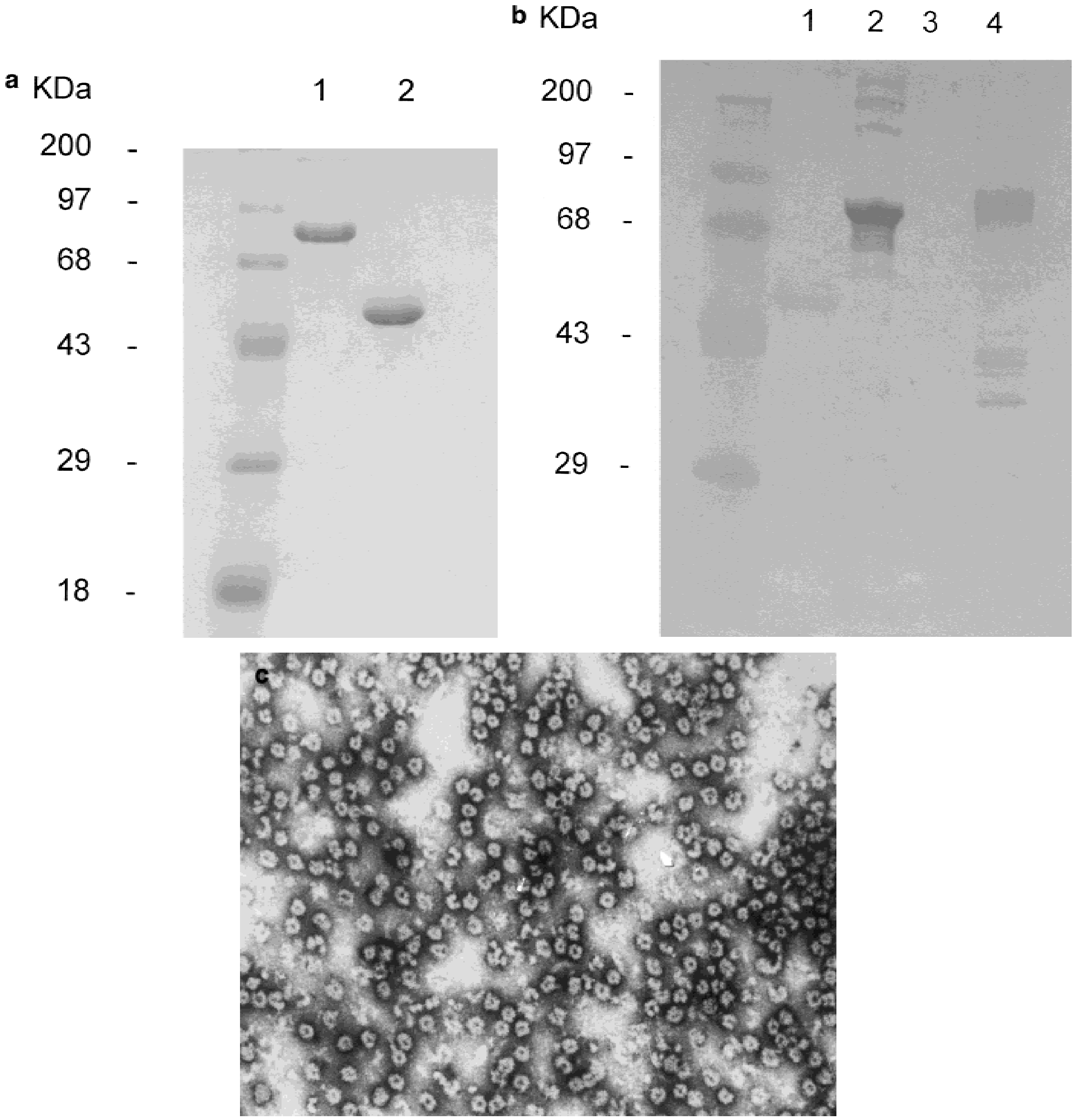

Figure 1. **a:** SDS polyacrylamide gel showing the expression of AP:Ty-VLPs fusion protein (*lane 1*) and Ty-VLPs (*lane 2*). **b:** Western blot analysis of Ty-VLPs (*lane 1*), AP-VLPs (*lane 2*), control uninfected cell lysate antigen (*lane 3*), and VZV-infected cell lysate antigen (*lane 4*). The blot was probed with polyclonal serum from a recently infected chickenpox patient (dilution 1:100). **c:** Electron micrograph of VLPs containing the AP of VZV. ×32,500.

The cultures were pulsed with tritiated methyl-thymidine (^{3}HTdR, Amersham International, Amersham, Bucks, UK. TRK120; 0.5 μCi/well; 40 μl) on day 5; after 16–18 hr the cells were harvested (TomTec Cell Harvester, Wallac, Milton Keynes, Bucks, UK) onto fibreglass filtermats. The thymidine incorporation was measured in a liquid scintillation counter (1450 Microbeta, Wallac) and expressed as the mean stimulation index (S.I.; sample cpm divided by background cpm) of triplicate cultures.

RESULTS

Cloning and Expression of Hybrid AP-VLPs

A vector containing AP of VZV was constructed for the production of AP-VLPs in yeast (pOGS1217). DNA encoding residues 304–605 of ORF 33 was inserted into the *Bam*HI site of pOGS40, the expression vector for Ty p1 [Adams et al., 1991]. This plasmid was transformed into yeast, and expression of the fusion protein, p1-AP was induced with galactose (500 ml culture). The yeast cells were homogenized and the debris removed by centrifugation. The clarified homogenate was centrifuged onto a sucrose cushion and the AP-VLPs further purified on a second sucrose gradient. VLPs were run on a reducing SDS-PAGE gel to confirm the presence of the p1-AP fusion protein. AP-VLPs were then produced at 12-liter scale and purified as previously described [Burns et al., 1991].

Figure 1a shows the location of hybrid AP-VLPs (lane 1) and control VLPs (lane 2) in a discontinuous

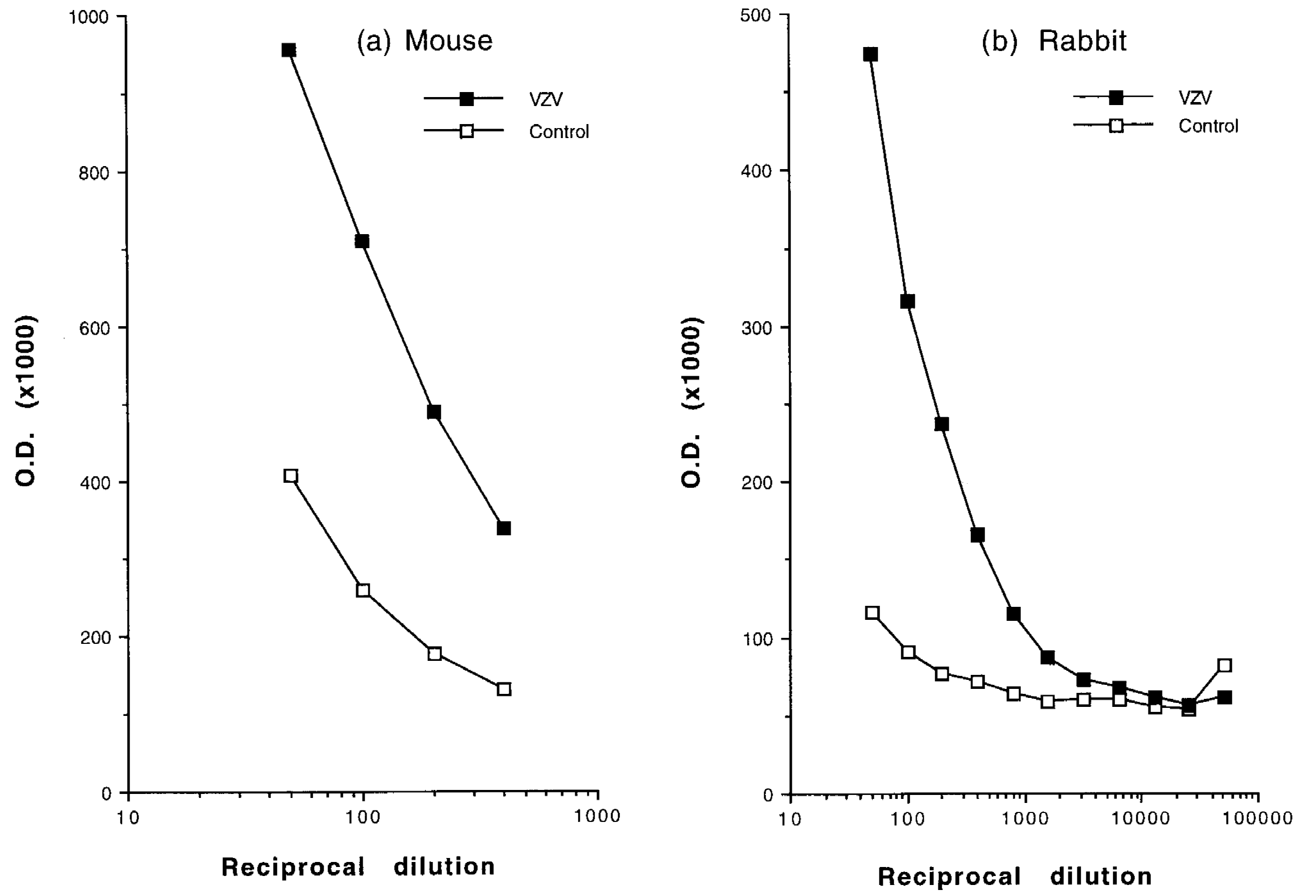

Fig. 2. **a:** Mouse anti-VZV antibodies determined by ELISA. Swiss white mice were immunised (2×) with AP-VLPs in alum. The serum represents a pool of 11 mouse sera. The pooled serum was tested on VZV-infected cell lysate antigen and control uninfected cell lysate antigen. **b:** Rabbit anti-VZV antibodies determined by ELISA. A Dutch dwarf rabbit was immunised (3×) with AP-VLPs in alum. The serum was tested on VZV cell antigen and control antigen.

SDS–PAGE gel. The molecular weight of the monomeric p1 protein in the control VLPs coincided with that described previously (Adams et al., 1987b) and runs at about 50 kDa, while the fusion p1-AP protein runs, as expected, with an approximate molecular weight of 79 kDa.

Western blot analysis of control VLPs, AP-VLPs, control antigen, and native VZV antigen with a serum from an individual with chickenpox is shown in Figure 1b. The chickenpox serum reacted very strongly with the AP-VLPs at 79 kDa and also with multimers of the p1-AP fusion protein. Very low reactivity (due to weak antihuman IgG-peroxidase conjugate binding) was observed with control VLPs and no reactivity was observed with the control antigen (uninfected MRC-5 cell lysate). The serum detected several bands on the VZV antigen, including a broad band at 75–83 kDa, containing the major membrane glycoproteins, and also two faint high-molecular-weight bands at 140/160 kDa. Other discernable bands were at 50, 42, 39, 38, 37, 34, and 33 kDa. The mAb 251D9, which reacts with the p32/p36 complex of the native virus [Friedrichs and Grose, 1986], did not react with AP-VLPs and only 2/10 sera from individuals with chickenpox reacted with AP-VLPs by Western blot analysis and enzyme-linked immunosorbent assay (ELISA) (data not shown).

The particulate nature of AP-VLPs was confirmed by electron microscopy (Fig. 2c)/ The electron micrograph shows purified AP-VLPs containing a dark core and a pale outer shell. The AP-VLPs are about 60 nm in diameter and look similar to, but larger than, the wild-type particles described previously [Mellor et al., 1985; Adams et al., 1987a].

Immunogenicity of AP-VLPs

In order to study the immunogenicity of the assembly protein, a group of 11 Swiss white mice and a Dutch dwarf rabbit were immunised with AP-VLPs as described under Materials and Methods. Figure 2 shows the anti-VZV antibody response, as measured by direct ELISA, present in sera from these animals. Good titres of VZV-specific antibodies were detected in both species. Mouse and rabbit anti-AP-VLP sera were tested in neutralisation assays (see Methods) but did not neutralise virus infectivity either in the presence or absence of complement (data not shown).

To study the T-lymphocyte response to the assembly protein, four different inbred mouse strains—BALB/c,

TABLE I. Proliferative Response of Lymph Node Cells from Inbred Mice Immunised With AP-VLPs

Antigen/ mitogen	μg/ml	Stimulation indices Mouse strain BALB/c	C57BL/6	CBA	B10.G
Con A	5	399.2[a]	11.5[a]	43.5[a]	35.9[a]
VLPs	10	123.0[a]	12.1[a]	53.7[a]	37.8[a]
Control	10	0.8	1.3	0.4	0.3
VZV	10	0.5	4.4[a]	3.1[a]	13.3[a]
VZV	1	0.5	0.9	1.8	14.2[a]
VZV	0.1	0.6	0.4	0.8	2.4[a]

[a]Stimulation index of >2.0 considered positive.

C57BL/6, CBA, and B10.G (H-2 haplotypes d, b, k, and 1, respectively)—with AP-VLPs (100 μg/mouse in alum, subcutaneously). At 7–14 days later, the inguinal lymph nodes were removed and the cells restimulated in vitro for 6 days with either VZV-infected cell antigen, control antigen, or control VLPs. Lymphocyte proliferation was measured by tritiated thymidine incorporation. The stimulation indices (S.I.) for these four strains are shown in Table I. Three (B10.G, CBA, and C57BL/6) out of four mouse strains produced a T-cell response recognising VZV.

B10.G mice had the strongest response to VZV (highest S.I. value of 13.3). The proliferative response to VZV was dose-dependent. All four strains responded to control VLPs and T cells from four strains responded to the mitogen, Con A.

Identification of the Products of VZV ORF 33

The mouse and rabbit sera raised against AP(305–605) were used for Western blot analysis of the VZV antigen preparation from infected cells. The sera raised in mice (Fig. 3a) and the rabbit (Fig. 3b) reacted with six discernible bands. The most intensely reactive bands were at 38 and 34 kDa. In addition, another four major reactive proteins were identified at 66, 63, 41, and 31 kDa (Table II). How these bands may relate to the predicted products of VZV ORF 33 is also shown in Table II. The 66 kDa band (translation product 1) is likely to represent the full-length VZV ORF33. The 41-kDa band is the product of the protease autocleavage of the 66 kDa protein at amino acid 236 releasing the protease (25 kDa predicted) and the immature assembly protein (41 kDa predicted). The antisera will not recognise the protease. The immature form of the AP is then cleaved by the protease toward the C-terminus and 27 amino acids are released, resulting in the mature AP (38 kDa predicted). This is the dominant band seen at 38 kDa. The intense band at 34 kDa could be a second translation product or a second cleavage product from the 41 kDa protein. A proposed scheme for the translation products of ORF 33 and the maturational cleavage products is shown in Figure 4.

DISCUSSION

VZV assembly protein p32/p36 (nucleoprotein complex) has many analogies with herpes simplex virus type 1 (HSV-1), cytomegalovirus (CMV) and other her-

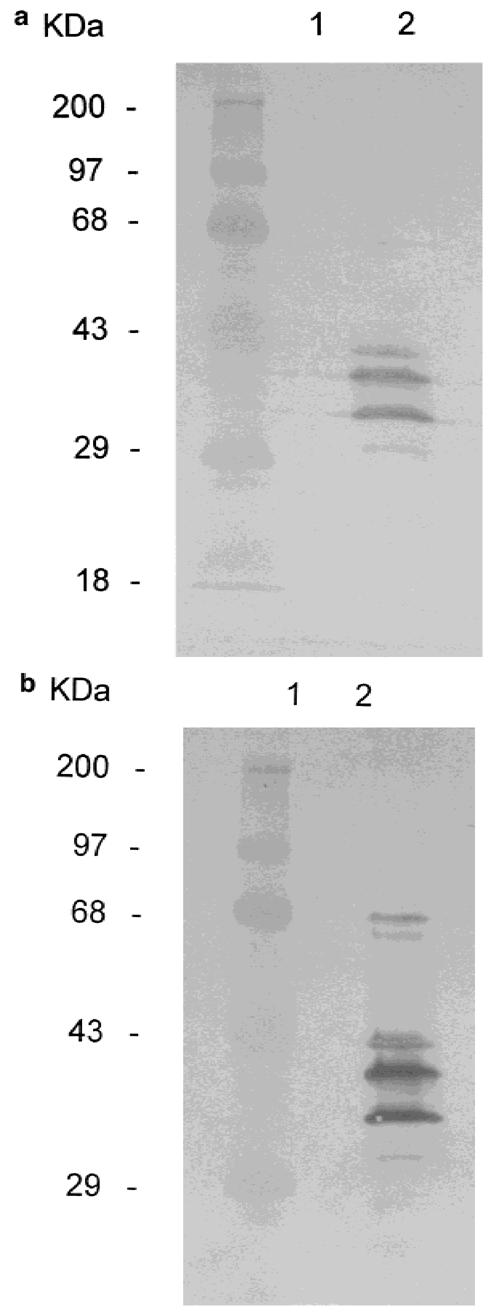

Fig. 3. Detection of VZV ORF 33 gene products. Western blot analysis using **(a)** mouse anti-AP-VLPs pooled serum (1:100 dilution) and **(b)** rabbit anti-AP-VLPS serum (1:100 dilution). *Lane 1,* control uninfected cell lysate antigen; *lane 2,* VZV-infected cell lysate antigen.

pesvirus assembly proteins [Welch et al., 1991; Harper, 1994]. The gene ORF 33 has been considered homologous to the HSV-1 assembly proteinase/protein gene (Davison and Scott, 1986). We produced recombinant AP(304–605)-VLPs in yeast to address two scientific questions; firstly to evaluate the antigenicity and immunogenicity of the AP protein and secondly to raise antisera with which to determine the maturation sequence of the assembly protein of VZV.

A serum from a patient infected recently with chick-

TABLE II. Products of Varicella-Zoster Virus ORF 33 in Infected Cells

Proposed product	Size by Western blotting[a] (kDa)	Immunoreactivity Mouse	Immunoreactivity Rabbit
Translation product 1 (TP1), assemblin	66	++	++
TP1 minus C-terminus, 27 residues	63	+	+
TP1 minus protease, carboxy-TP1 (C-TP1)	41[b]	++	++
C-TP1 minus C-terminus, 27 residues	38[b]	+++	+++
Translation product 2 (TP2)	34[b]	+++	+++
TP2 minus C-terminus, 27 residues	31	+/−	+/−

[a]Calculated using mouse anti-AP (304-605)-VLP serum.
[b]Doublets.

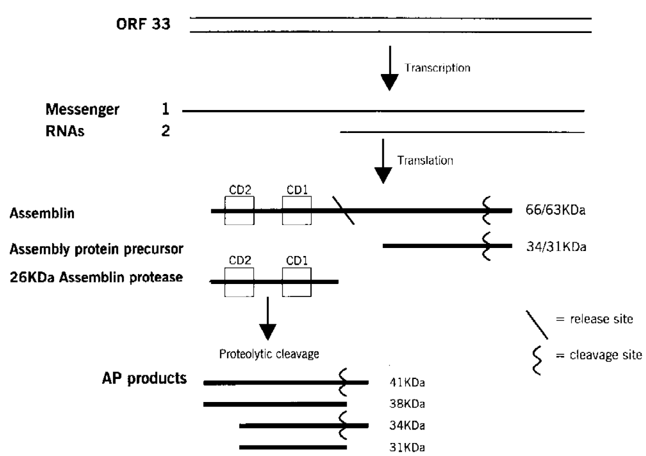

Fig. 4. Proposed translation products of VZV ORF 33 and maturational scheme for VZV AP based on Western blots of native VZV using sera from AP(304–605)-VLP immunised animals.

enpox allowed us to confirm the antigenicity of AP-VLPs. The serum reacted strongly with a band corresponding to the AP-VLPs and also to aggregation products of the p1-AP protein. IgM and IgG responses to the p32/p36 assembly protein complex have been studied previously [Harper and Grose, 1989] in chickenpox and zoster patients and also in individuals vaccinated with the live Oka vaccine. In these studies, the majority of sera showed strong IgM, but variable IgG responses to the complex. However, only a minority of sera from infected individuals reacted with AP-VLPs (IgG or IgM) and the mAb 251D9, which reacts with the p32/p36 complex of the native virus [Friedrichs and Grose, 1986], did not react with AP-VLPs by Western blot analysis or by ELISA, using the AP-VLPs coated to microtitre plates (data not shown). This indicates that the AP (p34) sequence as presented on VLPs is not generally antigenic for antibodies that recognise native p32/p36, even under reducing and denaturating conditions during SDS–PAGE analysis. This is somewhat of a paradox since AP-VLPs induce antibodies that can react with these proteins as discussed below.

The production of VLPs carrying fragments of the envelope protein gE of VZV was reported previously (Fowler et al., 1995) and gE-VLPS were found to be highly immunogenic in rodents and rabbits and a strong neutralising antibody response was produced. When mice and a rabbit were immunised with AP-VLPs, high levels of antibodies to VZV were detected by ELISA, but no neutralisation was observed with these sera from animals. This is to be expected, since AP is an internal protein inaccessible to antibodies.

Of more relevance to internal viral proteins is the T-lymphocyte response and both T-helper cell (CD4) and cytotoxic T-cell (CD8) responses can be generated to internal viral proteins, including VZV proteins-[Giller et al., 1989]. These cellular responses are vital in preventing viral infection and spread [Arvin, 1992, 1994]. We observed proliferative T-cell responses to VZV antigen in three out of four mouse strains immunised with AP-VLPs and the response was dose-dependent. It will be interesting to evaluate human CD4 and CD8 T-cell responses to AP during infection and these studies are in progress.

The sera raised against AP(304–605)-VLPs were used in Western blot analysis of native VZV virus and confirmed that the full-length protein (66 kDa, encoding both the protease and its substrate) is the primary translation product of the gene. The observed molecular weight was identical to the predicted molecular weight of 66.4 kDa [Davison and Scott, 1986]. The protease cleaves itself from the full-length protein at the previously predicted release site at residue 236 [Welch et al., 1991] resulting in two fragments: the assemblin protease (26 kDa) and the immature assembly protein (41 kDa). The 41-kDa protein was readily identifiable. The protease then cleaves off the C-terminal 27 amino acids from the immature AP, producing the mature AP at 38 kDa and also from the full-length protein, giving a protein of 63 kDa from which the protease can presumably release itself. The 38-kDa band was one of the two dominant bands with a 34-kDa protein that could represent either a second translation product from the ORF33 (residues 304–605) as proposed for the CMV assembly protein maturation (Welch et al., 1991a) or a cleavage product from the 41-kDa/38-kDa assembly proteins. The former case is more likely since there are no obvious second assemblin protease cleavage sites within the 41-kDa protein, although another enzyme could be active. The sixth band observed at 31 kDa could represent the 34-kDa protein minus its C-terminus 27 amino acids or a degradation product of the 41-kDa/38-kDa assembly proteins. Western blot analysis using a more sensitive chemiluminescence technique in a different laboratory confirmed the presence of these bands and their molecular weight.

Previous studies, using the mAb 251D9, have indicated that the predominant assembly proteins have molecular weights of 32 and 36 kDa; these two bands are termed the assembly protein complex [Friedrichs

and Grose, 1986; Harper and Grose, 1989]. These may correspond to the two major bands detected in our studies at p34/p38. It is interesting to note that the mAb recognising p32/p36 blots neither the full-length ORF33 product (66-kDa protein) nor the 41-kDa protease cleavage product, whereas the polyclonal antibodies raised with AP(304–605)-VLPs do. It is not clear why the mAb fails to pick up the 41-kDa precursor assembly protein and the AP(304–605)-VLPs, particularly since it detects smaller fragments presumably consisting of these consequences. The bands at 41, 38, and 34 kDa appear to be doublets with the major species being of a lower molecular weight. It is not clear, however, whether the higher-molecular-weight bands are derivatives of the main bands, for example, phosphorylated derivatives as previously identified for some assembly proteins [Ashcroft et al., 1993] or whether they are distinct protease cleavage products or translation products.

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